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Structure and Development of Neuronal Connections in Isogenic Organisms*: Transient Gap Junctions between Growing Optic Axons and Lamina Neuroblasts

(neuronal specificity/serial sections/crustacea)

V. LOPRESTI, E. R. MACAGNO, AND C. LEVINTHAL

Department of Biological Sciences, Columbia University, New York, New York 10027

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ABSTRACT **We previously showed that the growth of each bundle of eight optic fibers from one ommatidium into the optic lamina of Daphnia occurs in such a way that one of the eight fibers precedes the others into the lamina. The growth cone of this lead fiber makes surface contact with undifferentiated neuroblasts near the midplane. This is followed by a glial-like wrapping of each neuroblast around the fiber. In this report, gap junctions are shown to form for a short period of time between the growing lead fiber and the neuroblast that is wrapping around it. It is proposed that these junctions may represent a morphological correlate of informational exchange between axon and neuroblast. This signaling would then reflect the fact that the sequence of axon proliferation by the lamina neuroblasts within an optic cartridge, ultimately composed of five lamina neurons and eight optic fibers, parallels the order in which the neuroblasts undergo the wrapping reaction with the lead fiber.**

In previous reports, we followed the overall pattern of optic nerve growth and interaction with eventual postsynaptic cells in the optic lamina of Daphnia using the technique of three-dimensional reconstruction from serial sections (1–3). It was shown that each first-order synaptic unit is a type of optic cartridge (4), consisting of the eight optic fibers from the retinula cells of an ommatidium and five optic lamina neurons. The general pattern of synapses among the pre- and postsynaptic elements of the unit is essentially constant among several genetically identical adults. However, it shows some variation in the absolute number of synapses between any two elements. Each cartridge is initially organized by the precocious growth of only one of the eight optic axons of an ommatidium, which we have called the "lead" fiber. The growth cone of this lead fiber was observed to travel close to the midplane, sucessively making contact with five morphologically undifferentiated optic lamina neuroblasts. After the initial touch by the growth cone, each neuroblast wraps around the lead axon in a glial-like fashion. This interaction is transient but always basically the same for a particular cell at a given developmental stage. We have undertaken a detailed ultrastructural study of the interaction of growing optic axons with lamina neuroblasts, and we report here the existence of gap junctional contacts between lead fiber and lamina neuroblast at the wrapping stage, hours before the onset of synaptogenesis in the cartridge.

The small number of cells present in the eye and optic ganglion of Daphnia have previously enabled us to identify the same optic cartridges in embryos of different stages, and thus to follow individually identified cells and fibers as the morphogenesis of a synaptic unit proceeds. This capability led to the observation that the five initially undifferentiated neuroblasts of a forming cartridge undergo morphological differentiation in a precise order. This order reflects the sequence in which the neuroblasts were touched and participated in the glial-like reaction with the lead fiber. The sequential axon proliferation by these second-order neurons caused us to postulate the existence of some form of informational flow from optic fiber to neuroblast that might trigger or instruct its differentiation. The present study has revealed the existence of gap junctional contacts between the two elements, similar to those that have been shown to provide routes of exchange of molecules at least as large as 300 molecular weight, and to mediate electrical coupling in both embryonic and adult tissues (5–9). As far as we have determined, the growing axon-neuroblast junctions are confined to the wrapping area, and are similarly transient in nature. This report deals with the observation of these particular junctions in optic cartridge morphogenesis, and their possible significance.

The ultrastructural study has also revealed the existence of gap junctions both between the growth cones of lead fibers and glial cells, and between lamina neurons and glial cells. The first of these are limited in occurrence and are of small size, while the second are large and seemingly ubiquitous. They occur between glia and lamina neurons at all medio-lateral levels of the lamina and, thus, at widely different stages of lamina cell development (3). The significance of these classes of junctions is presently unclear. The study has also shown the presence of a large number of desmosomal contacts among all growing fibers from one ommatidium, and between the fibers and their cartridge neuroblasts throughout the contact area. It thus appears that the elements of the developing unit are structurally bound together during morphogenesis. Finally, another possible route of informational exchange is indicated by the occurrence of what appears to be a process similar to phagocytosis. Serial sections reveal both coated and uncoated membrane-bound vesicles which seem to be in the process of "pinching-in." These vesicles contain cytoplasmic fingers of a fiber or cell apparently being engulfed by another cartridge element. Fully closed vesicles containing membrane-bound cytoplasmic inclusions are also seen, but these cannot be related to those in formation without the use

* This paper is number 3 in a series. Numbers 1 and 2 are references 2 and 3, respectively.

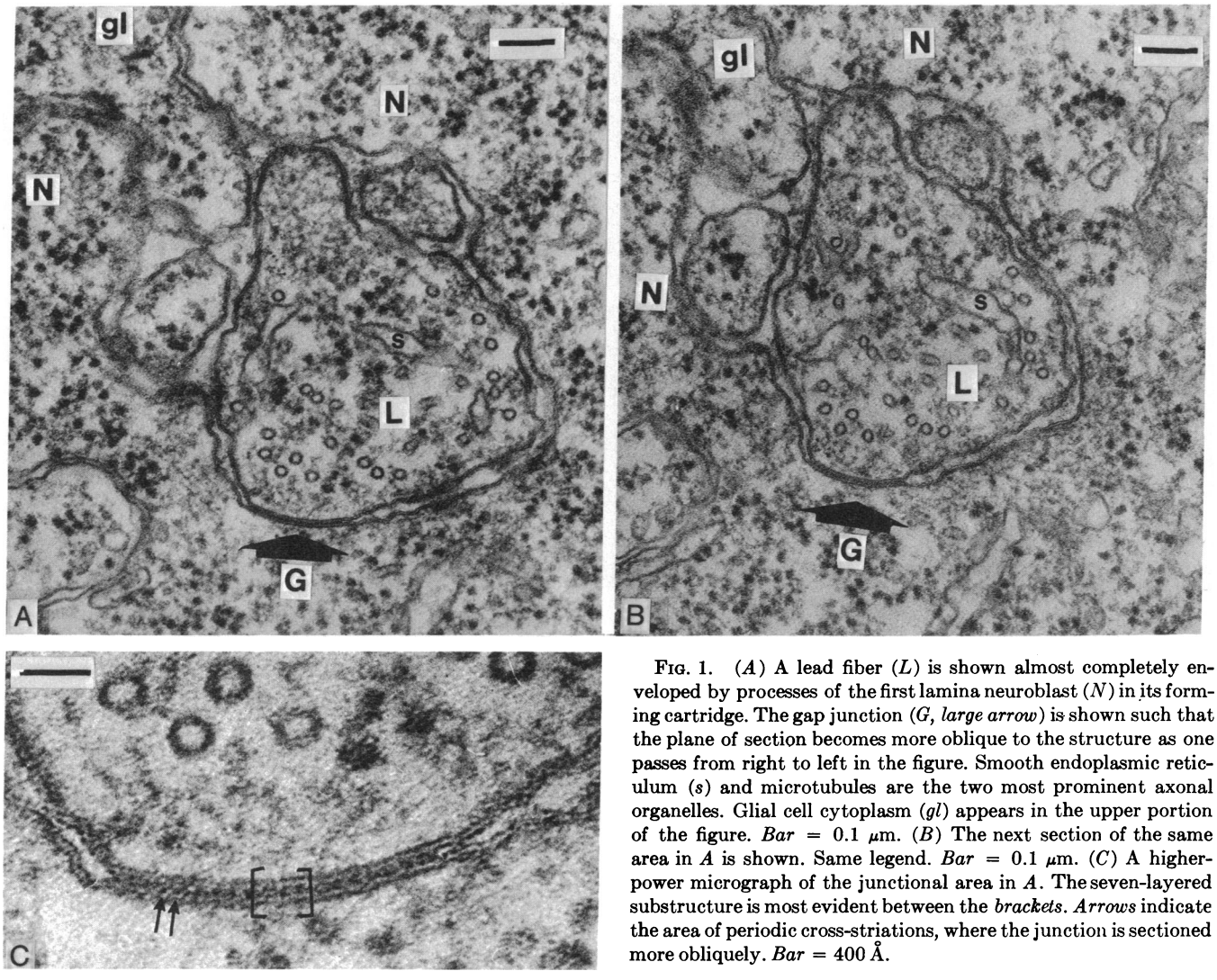


FIG. 1. (*A*) A lead fiber (*L*) is shown almost completely enveloped by processes of the first lamina neuroblast (*N*) in its forming cartridge. The gap junction (*G, large arrow*) is shown such that the plane of section becomes more oblique to the structure as one passes from right to left in the figure. Smooth endoplasmic reticulum (*s*) and microtubules are the two most prominent axonal organelles. Glial cell cytoplasm (*gl*) appears in the upper portion of the figure. *Bar* = 0.1 μm. (*B*) The next section of the same area in *A* is shown. Same legend. *Bar* = 0.1 μm. (*C*) A higher-power micrograph of the junctional area in *A*. The seven-layered substructure is most evident between the *brackets*. *Arrows* indicate the area of periodic cross-striations, where the junction is sectioned more obliquely. *Bar* = 400 Å.

of cytochemical tracer techniques. These techniques are presently being applied to attempt to trace the formation and fate of these inclusions.

MATERIALS AND METHODS

Staging of embryos and development *in vitro* were as described (3). For fixation, a number of different sequential aldehyde–osmium procedures were tried. Initially, the greatest problem was the speed of penetration of any of the fixatives, as the embryo is a closed system with large yolk reserves that tend to act as another penetration barrier. The problem was solved by dissection of the head away from the rest of the body and removal of as much yolk as possible under a dissecting microscope, immediately after immersion into ice-cold primary fixative. The procedure arrived at is as follows: embryos of the relevant stages are dissected into 3% glutaraldehyde in 35 mM phosphate buffer, pH 7.4, for 25 to 30 minutes. They are then immediately transferred without any buffer rinse to a mixture of 3% glutaraldehyde, 0.5% osmium tetroxide in the same buffer for 1 hour, briefly rinsed twice in 50 mM phosphate buffer, pH 7.4, twice in 50 mM sodium maleate buffer, pH 5.9, and bloc-stained for 1 hour in freshly prepared 2% uranyl acetate in the latter buffer. All steps were performed at 0°C, as was the subsequent dehydration in a graded acetone series. Specimens were embedded in Epon 812. The key to obtaining trilaminar membrane images was the use of the glutaraldehyde–osmium mixture as a postfix rather than buffered osmium alone. (This mixture as a primary fixative yielded equally good membrane preservation, but intracellular preservation was inferior.) Times significantly longer than 30 minutes in primary fixative were detrimental; the procedure was ineffective if the primary fixation was done at room temperature, yielding unilaminar membrane images. To insure that the extracellular spaces were not being artificially widened or narrowed, about ten variations in osmolarity were tried before the final solutions were determined. Many of the procedures that were not useful in the embryo gave satisfactory preservation in adult Daphnia.

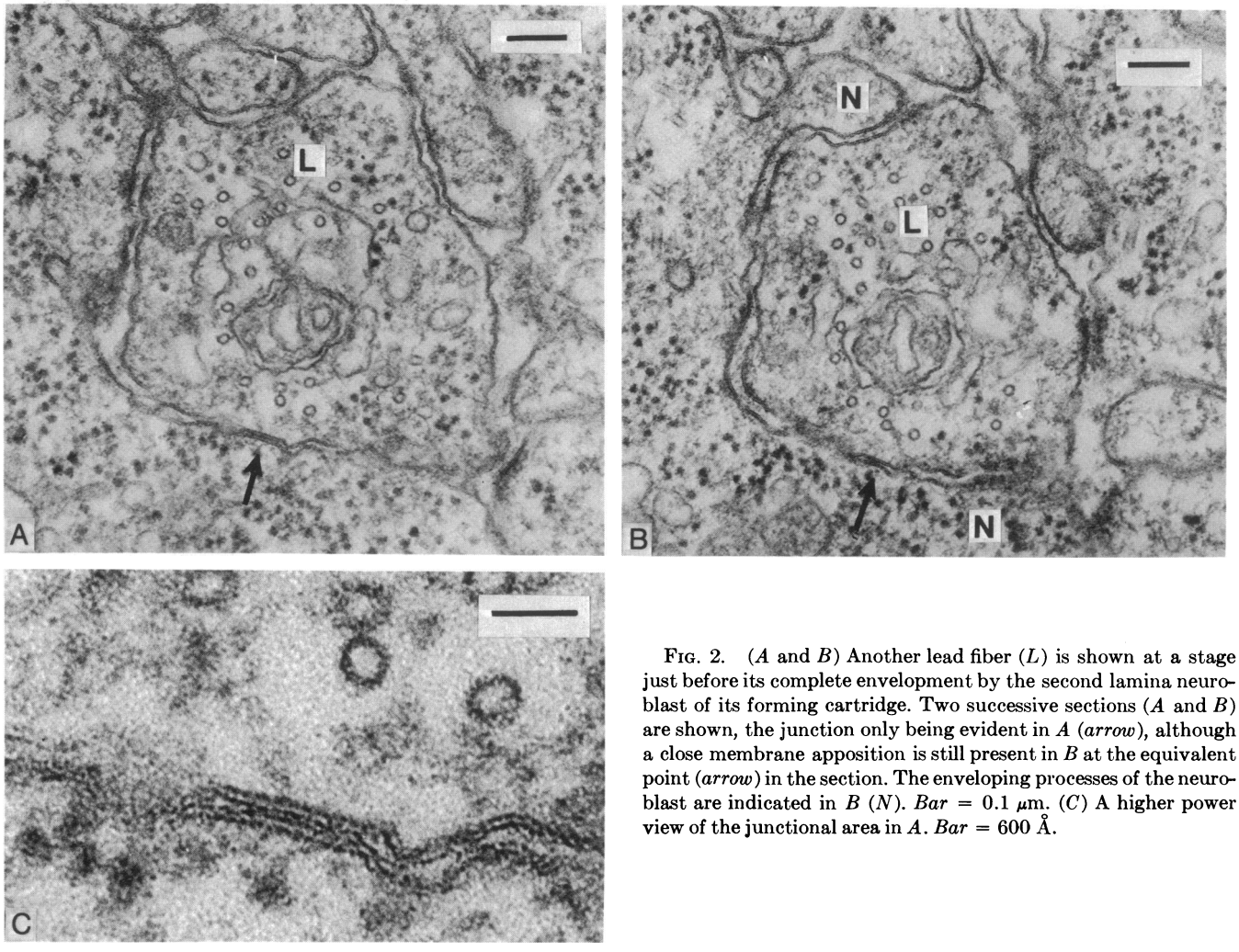


FIG. 2. (*A* and *B*) Another lead fiber (*L*) is shown at a stage just before its complete envelopment by the second lamina neuroblast of its forming cartridge. Two successive sections (*A* and *B*) are shown, the junction only being evident in *A* (*arrow*), although a close membrane apposition is still present in *B* at the equivalent point (*arrow*) in the section. The enveloping processes of the neuroblast are indicated in *B* (*N*). *Bar* = 0.1 μm. (*C*) A higher power view of the junctional area in *A*. *Bar* = 600 Å.

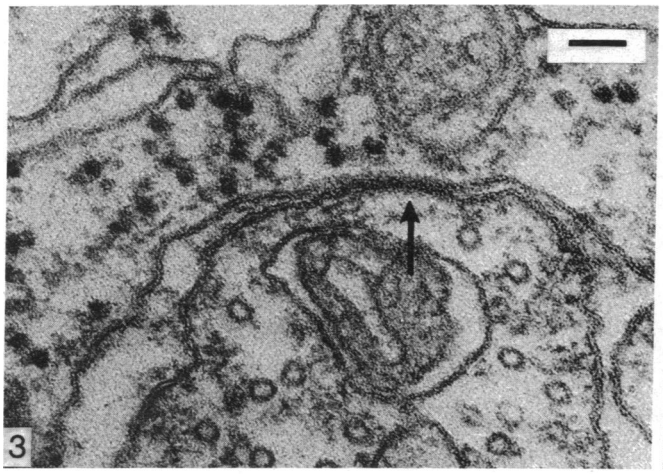
Serial sections were cut at 500 Å (gray by reflected light) on a Sorvall MT-2B ultramicrotome. Approximately 500 such serial sections per specimen were collected on 1 × 2-mm oval grids, coated with formvar (0.4% in chloroform) and a light coat of evaporated carbon, and spanning the area from the base of the eye sufficiently far into the lamina to insure that the relevant volume of lamina was included in its entirety. No more than five sections in each series were lost, with no two of the missing sections consecutive. Grids were stained for 1.5 hours at 37°C in aqueous 7% uranyl acetate which was filtered before use. Lead staining was not used as it tends to obscure the seven-layered structure of the junctions and contributes to an increased granularity in the image. The first 400 sections in each series were scanned at 40,000 to 60,000 magnification on a Siemens Elmiskop 101, and photographed at 80 kV.

RESULTS

The results of this study derive mainly from the scanning of seven lead fibers in two different embryos of slightly different ages. The fibers studied were those of forming optic cartridges 9, 10, and 11 in each lobe of the lamina (see ref. 3 for numbering system) at the time of their wrapping interaction with either neuroblasts 1, 2, or 3 or their cartridges.

Figs. 1 and 2 show gap junctions in two different lead fibers at slightly different stages of their growth progression. In Fig. 1, the glial-like wrapping is just about at its maximal extent, and the cell involved is neuroblast 1 (the most anterior and first to be touched) of the eventual optic cartridge. Two serial sections of the junction are shown (Figs. 1*A* and 1*B*); it appears in five consecutive sections, having an area of about 0.2 μm^2. In Fig. 2, the lead fiber is shown in two consecutive sections (Figs. 2*A* and 2*B*) at the early interaction stage with neuroblast 2 of its forming cartridge. Fig. 2*B* shows that at this stage, the lead axon is not completely enveloped as in Fig. 1, but is exposed to glial-cell cytoplasm on one side. Correspondingly, the gap junctional area has the appearance of being in the process of formation. The image in Fig. 2*A* appears in only two sections, the second not shown, while the bracketing sections appear as in Fig. 2*B*. It is to be noted that Fig. 2*A* presents the junction in cross section, showing a seven-layered structure, with the outer leaflets of the apposed membranes separated by about a 20-Å gap. By contrast, in Fig. 1, the junction is sectioned at a more oblique angle, so that an approximate 100-Å periodicity of the cross-channel elements can be discerned in some areas (10, 11).

In the remaining five lead fibers scanned, two additional fibers showed areas of either close membrane apposition with



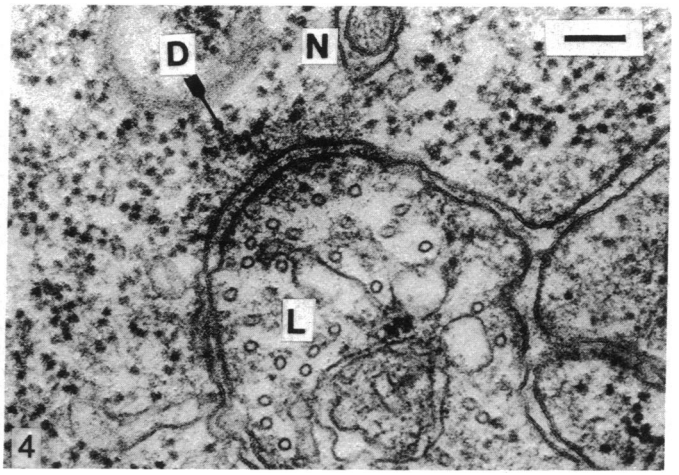


FIG. 3. A possible junctional area is shown (*arrow*) such that the substructure cannot be distinguished at this sectioning angle. *Bar* = 500 Å.

FIG. 4. A lead fiber (*L*) is shown making a desmosomal contact (*D*) with one of its neuroblasts (*N*). *Bar* = 0.1 μm.

the surrounding neuroblast in two or three consecutive sections, or as shown in Fig. 3, areas where there appeared to be a junction sectioned at an angle such that its substructure could not be resolved by present methods. These elements were classed as possible forming or formed junctions. Application of lanthanum hydroxide (12) should allow visualization of junctions sectioned in the transverse plane, although freeze cleaving will probably not be useful due to the rarity and specific localization of these structures. Finally, in the remaining three lead fibers, although there occurred areas of close membrane apposition in isolated sections, no strong evidence for gap junctions was inferred. These findings are subject to two main interpretations: either the occurrence of these structures at the wrapping stage is not a universal event, or else it is universal but extremely transient in nature, such that the probability of finding a completely formed structure combined with the probability of sectioning it close to cross section is small. As we have never found axon–neuroblast junctions outside the wrapping area, and given the stereotypic reproducibility of the wrapping interaction, also a transient event now seen for at least the first four cells of forming cartridges, we favor the latter interpretation as discussed below.

Another significant membrane specialization found in forming cartridges is a desmosome-like attachment, characterized by a space of 200 Å between apposed membranes, with a fine filamentous network spanning the intercellular space and carrying through both membranes (Fig. 4). These structures are numerous and are not confined to any particular area, but are distributed all along the contact area between the lead fiber and its associated neuroblasts, including the wrapping area and the region of the growth-cone surface contact. In more proximal areas of the series, where the ends of follower fibers are present, desmosomes are found among all growing processes in the bundle from one ommatidium, as well as between followers and neuroblasts where they have grown to this point. We interpret these organelles as merely structurally binding together all elements of the forming cartridge, no informational role having ever unambiguously been ascribed to desmosomes.

DISCUSSION

The study of the three-dimensional morphology of individual forming optic cartridges at different developmental stages has led to the observation that the morphological differentiation of the five lamina neuroblasts in each cartridge occurs in a sequential fashion, paralleling the order of their association with growing optic fibers. In principle, several alternative mechanisms can be postulated. First, it is possible that the onset of morphological differentiation by lamina neuroblasts is entirely determined by their mitotic history; for example, some fixed interval, beginning from the time of final mitosis, might be required for the synthesis of new proteins needed for axon proliferation. The order of axon formation among the cells of a cartridge would then depend on the order in which the cells finished their last division. Second, the cells might respond to environmental cues to differentiate, on the basis of their antero-posterior order in the midplane, perhaps due to a chemical gradient of the same type which we think of as being involved in restricting new lead fibers to midplane growth. Finally, the sequential differentiation of the neuroblasts may be a function of their interactions with the lead fiber, and such interactions could serve either to trigger a preset program of differentiation, or perhaps even play an instructive role. Regarding this final possibility, it is important that the distinction be made between inductive and instructive information. An inductive signal would be confined merely to conveying information to the lamina neuroblast that it should now begin the final biochemical steps leading to the formation of its nerve fibers, but no information as to what these steps might be. The assumption is that this information is inherent in the developmental genetic program of the cell. The results of this type of information might in themselves be sufficient in ordering the somewhat later synaptogenesis. An instructive signal, on the other hand, might involve the passage of information that would, for example, biochemically label the neuroblast as a specific one of the five postsynaptic cells that comprise a single cartridge. In this model one would assume that the information transfer would provide the biochemical label that would in turn lead to a defined morphology

for the growing axon. However, molecules with a high information content are not known to pass through gap junctions.

We cannot as yet suggest any relevant evidence to distinguish between induction or instruction in our system. It seems to us, however, that information transfer of some kind is favored over other alternatives by the occurrence of gap junctional contacts at the stereotypic wrapping interaction. The alternative possibilities can perhaps only be distinguished by preventing the growth of a lead fiber and examining the subsequent events in the developing lamina.

A number of observations in both vertebrates and invertebrates indicate that similar interactions may exist between growing sensory axons and their second-order central neurons. In Xenopus, the work of Jacobson and coworkers (13) has established that before axon proliferation, retinal ganglion cells have acquired spatial information such that each cell possesses a unique address in the developing retina. The problem still remains as to how this property is transmitted to tectal cells during optic nerve invasion of the tectum at stages 37 to 39. Observations of LaVail and Cowan in the chick (14) suggest that outer laminae of the tectum do not become well organized for some days after the initial ingrowth of ganglion cell axons, and this process of tectal laminar organization is severely limited if the eye is removed before optic nerve growth. Similarly, Hinds (15) has observed that in developing mouse olfactory bulb, mitral cells, which are the primary synaptic sites for olfactory axons, are seen to possess the beginnings of mature tangential axonal processes only in regions where olfactory nerve axons have previously penetrated. This observation, combined with the fact that extirpation of the olfactory placode before olfactory nerve outgrowth in larval urodele amphibians (16) results in a forebrain devoid of secondary and tertiary olfactory tracts, and presumably therefore of mitral cells, leads Hinds to conclude that olfactory nerve axons play a crucial role in eliciting olfactory bulb differentiation.

In invertebrates, Trujillo-Cénoz and Melamed (17) have demonstrated in a serial section study at the electron microscope level that the early growth of retinula axons in muscoid flies results in a transient direct uncrossed retina-lamina projection before the formation of the crossed adult type of projection characteristic of unfused eyes. An intermediate rearrangement thus determines the formation of the final optic cartridges. At the initial primitive projection, the authors find large numbers of desmosome-like contacts but no evidence of synaptic contacts, and they speculate as to the possibility that this transient projection may serve a "neuromorphogenetic function." Observations on both the highly-ordered temporal sequence of axon outgrowth from retinula cells of *Drosophila*, together with similar results on the reorganization of the initial retina-lamina projection, have led Hanson (18) to postulate an induction of second-order cells in the developing lamina.

In Daphnia, the number of retinula and lamina neurons is an order of magnitude smaller than in flies. This allows us to look at the growing optic system as a whole, and permits us to move one step further and examine the steps involved in the differentiation of single identified cells at different developmental stages. The present results strongly support the conclusion that there are specific events at well-defined times in the formation of a cartridge by which the growing lead axon exerts some influence on the differentiation of the eventual post-synaptic cells of its unit. Since the lead fiber, like the other fibers from the ommatidium, only makes extensive synaptic contacts with one of the five lamina neurons of its cartridge, the signalling event is clearly not limited to its particular synaptic sites. The lead fiber however, is to our knowledge the only one of the eight that undergoes the interaction reported here. The nature of the proposed signalling event, whether it consists of an inductive triggering effect or an instructional exchange, and its consequences in terms of the actual process of specific synaptogenesis, remain to be determined.

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